BASIC AND TRANSLATIONAL—BILIARY

MicroRNA-26a Promotes Cholangiocarcinoma Growth by Activating β-catenin

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BACKGROUND & AIMS: MicroRNAs (miRNAs) have been implicated in the development and progression of human cancers. We investigated the roles and mechanisms of miR-26a in human cholangiocarcinoma. **METHODS:** We used in situ hybridization and quantitative reverse transcriptase polymerase chain reaction to measure expression of miR-26a in human cholangiocarcinoma tissues and cell lines (eg, CCLP1, SG231, HuCCT1, TFK1). Human cholangiocarcinoma cell lines were transduced with lentiviruses that expressed miR-26a1 or a scrambled sequence (control); proliferation and colony formation were analyzed. We analyzed growth of human cholangiocarcinoma cells that overexpress miR-26a or its inhibitor in severe combined immune-deficient mice. Immunoblot, immunoprecipitation, DNA pull-down, immunofluorescence, and luciferase reporter assays were used to measure expression and activity of glycogen synthase kinase (GSK)-3β, β-catenin, and related signaling molecules. **RESULTS:** Human cholangiocarcinoma tissues and cell lines had increased levels of miR-26a compared with the noncancerous biliary epithelial cells. Overexpression of miR-26a increased proliferation of cholangiocarcinoma cells and colony formation in vitro, whereas miR-26 depletion reduced these parameters. In severe combined immune-deficient mice, overexpression of miR-26a by cholangiocarcinoma cells increased tumor growth and overexpression of the miR-26a inhibitor reduced it. *GSK-3β* messenger RNA was identified as a direct target of miR-26a by computational analysis and experimental assays. miR-26a–mediated reduction of GSK-3β resulted in activation of β-catenin and induction of several downstream genes including *c-Myc*, *cyclinD1*, and *peroxisome proliferator-activated receptor δ*. Depletion of β-catenin partially prevented miR-26a–induced tumor cell proliferation and colony formation. **CONCLUSIONS: miR-26a promotes cholangiocarcinoma growth by inhibition of GSK-3β and subsequent activation of β-catenin. These signaling molecules might be targets for prevention or treatment of cholangiocarcinoma.**

Keywords: Biliary Tract; Prostaglandin; COX-2; Post-Transcription Gene Regulation.

Cholangiocarcinoma is a highly malignant cancer of the biliary tract with a poor prognosis.[1–9] The incidence and mortality of cholangiocarcinoma is rising worldwide, and currently there is no effective chemoprevention or treatment. The tumor often arises from background conditions that cause long-standing inflammation, injury, and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis, clonorchiasis, hepatolithiasis, or complicated fibropolycystic diseases. The pathogenesis of cholangiocarcinoma is complex and involves alterations of a number of signaling cascades and molecules, including Wnt/β-catenin[10–14] and cyclooxygenase-2 (COX-2)-derived prostaglandin E_2 (PGE_2) pathways.[5]

β-catenin is a key mediator in Wnt regulation of multiple cellular functions in embryogenesis and tumorigenesis.[15–18] In the absence of a Wnt signal, β-catenin exists within a cytoplasmic complex (β-catenin destruction complex) along with glycogen synthase kinase 3β (GSK-3β), adenomatous polyposis coli, and axin, where it is phosphorylated and targeted for degradation by the proteasome. Activation of Wnt signaling disrupts this destruction complex, leading to cytoplasmic accumulation of β-catenin and allowing its translocation into the cell nucleus. In the nucleus, β-catenin associates with T-cell factor (TCF)/lymphoid enhancer factor (LEF) that stimulates transcription of target genes important for proliferation, differentiation, and apoptosis.[15–18] Recent studies have shown that accumulation of nuclear β-catenin is induced by COX-2/PGE_2, in addition to the canonical Wnt/Frizzled signaling, in human colon cancer cells[19,20] and cholangiocarcinoma cells.[10]

MicroRNAs (miRNAs) are noncoding RNAs of 20–22 nucleotides involved in the regulation of gene expression at a post-transcriptional level by binding to the target sites of messenger RNAs (mRNAs). Recent studies suggest an important role of miRNAs in a number of human and animal cancers[21,22]; however, the potential implication of miRNAs in cholangiocarcinogenesis remains largely unknown. Given the recently documented importance of miRNAs in protein

Abbreviations used in this paper: COX-2, cyclooxygenase-2; CTD, C-terminal domain; GSK-3β, glycogen synthase kinase-3β; LEF, lymphoid enhancer factor; miRNA, microRNA; mRNA, messenger RNA; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE_2, prostaglandin E_2; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SCID, severe combined immune deficienct; siRNA, small interfering RNAs; TCF, T cell factor; UTR, untranslated region.

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regulation and tumorigenesis, we postulated that GSK-3β/β-catenin pathway might be regulated by miRNAs during cholangiocarcinoma growth. To explore this possibility, we performed computational analysis using TargetScan 5.1 and this approach led us to identify miR-26a as a noncoding RNA that directly binds to the 3′-untranslated region (UTR) of the GSK-3β mRNA. The objective of the current study was to validate the effect of miR-26a on GSK-3β in cholangiocarcinoma cells and to examine the role of this mechanism in cholangiocarcinogenesis and tumor progression. Our findings demonstrate a novel role of miR-26a-mediated β-catenin activation in human cholangiocarcinoma.

Materials and Methods

In situ hybridization for miR-26a was performed in the formalin-fixed and paraffin-embedded tissue specimens surgically resected from patients diagnosed with cholangiocarcinoma by using the MiRCURY LNA microRNA ISH Optimization Kit (Exiqon, Vedbaek Denmark), with the approval of the Institutional Review Board.

Four human cholangiocarcinoma cell lines, including CCLP1,[23] SG231,[24] HuCCT1,[25] and TFK1,[26] and 1 noncancerous cholangiocyte cell line (H69) were used in this study. The CCLP1, SG231, and HuCCT1 cells were cultured according to our methods as described previously.[10,27–29] The TFK1 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin as described previously.[26] The H69 cells were cultured in Bronchial Epithelial Cell Basal Medium (Lonza, Basel, Switzerland) supplemented with growth factors in BEGM SingleQuot Kit and 10% heat-inactivated fetal bovine serum.

Human cholangiocarcinoma cell lines were transduced with miR-26a1 lentivirus or miRNA-scramble control lentiviral vector and the cells were analyzed for proliferation and colongenic formation.

A severe combined immune-deficient (SCID) mice tumor xenograft model was used to determine the effect of miR-26a on cholangiocarcinoma growth in vivo. The tumor xenografts were established by inoculating 1.5×10^6 miR-26a-overexpressed or control CCLP1 cells into the flanks of mice (6 mice per group), and the animals were observed for 4 weeks for tumor formation.

Western blotting, immunoprecipitation, DNA pull-down, immunofluorescence, and luciferase reporter activity assays were performed to determine the expression and activity of glycogen synthase kinase-3β (GSK-3β)/β-catenin and related signaling molecules.

The methods are described in more detail in the Supplementary Material.

Results

Expression of miR-26a Is Increased in Human Cholangiocarcinoma Tissues and Cell Lines

Human cholangiocarcinoma tissue samples and non-neoplastic bile duct epithelia were subjected to in situ hybridization using locked nucleic acid-modified probe against miR-26a. Formalin-fixed, paraffin-embedded tumor and liver tissues from 21 patients who underwent surgical resections for cholangiocarcinoma were analyzed. In cholangiocarcinoma tissues, miR-26a is expressed in 19 of 21 cases (90.5%); in normal bile ducts and non-neoplastic peribiliary glands, miR-26a is expressed in 7 of 21 cases (33.3%). As shown in Figure 1*A* and Supplementary Table 1, the staining frequency and intensity of miR-26a is significantly higher in cholangiocarcinoma cells (19.0% +++, 28.6% ++, 42.9 +, 9.5% −) compared to the non-neoplastic bile duct epithelial cells (0% +++, 4.8% ++, 28.6% +, 66.7% −) ($P < .001$). Consistent with these observations, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis showed higher levels of miR-26a expression in 4 human cholangiocarcinoma cell lines (ie, CCLP1, HuCCT1, SG231, TFK1) compared to the noncancerous human biliary epithelial cell line H69 (Figure 1*B*). These findings provide novel evidence for overexpression of miR-26a in human cholangiocarcinoma tissues and cell lines.

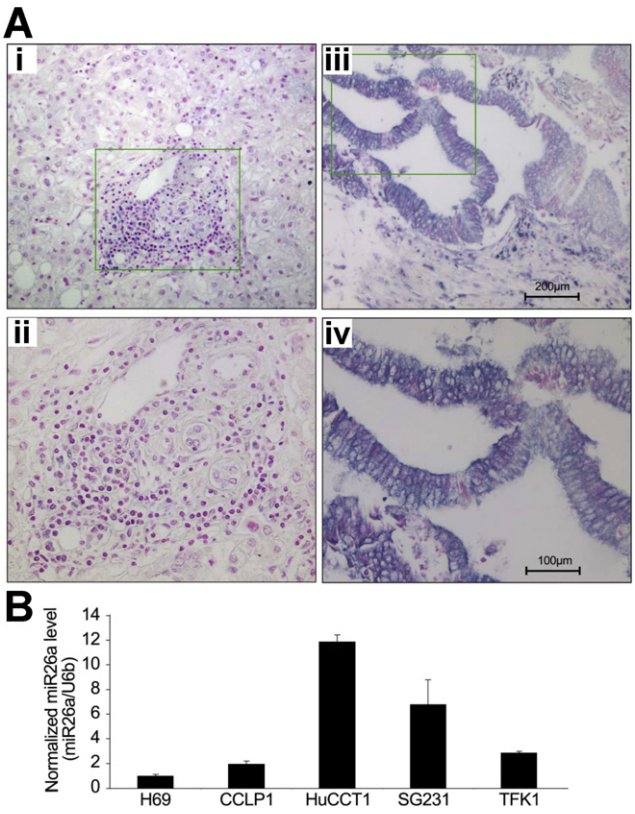

Figure 1. Expression of miR-26a in human cholangiocarcinoma tissues and cell lines. (*A*) In situ hybridization for miR-26a in human cholangiocarcinoma tissues was performed as described in the Materials and Methods section. Positive signals were shown as *dark blue*; nuclei were counterstained as *red*. (*i,ii*) Negative staining in the normal bile duct epithelial cells in the portal tracts; (*iii,iv*) positive miR-26a staining in human cholangiocarcinoma cells. *Panels ii* and *iv* (200×) represent high magnifications of *panels i and iii* (100×), respectively, from the highlighted areas. (*B*) qRT-PCR for mature miR-26a in a human cholangiocyte cell line (H69) and 4 human cholangiocarcinoma cell lines (ie, CCLP1, HuCCT1, SG231, TFK1). Results represent the mean ratio between miR-26a and the control miRNA U6b from 3 experiments.

miR-26a Promotes Cholangiocarcinoma Cell Growth, In Vitro

To investigate the role of miR-26a in cholangiocarcinoma cell growth, we constructed human cholangio-